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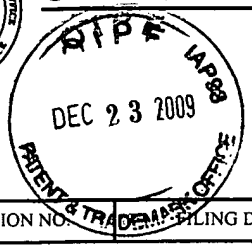

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/582,679	05/17/2007	Jo Klaveness	PN0397	7412

36335 7590 11/13/2009

GE HEALTHCARE, INC.
IP DEPARTMENT 101 CARNEGIE CENTER
PRINCETON, NJ 08540-6231

EXAMINER	
SCHLIENTZ, LEAH H	
ART UNIT	**PAPER NUMBER**
1618	
MAIL DATE	**DELIVERY MODE**
11/13/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/582,679	KLAVENESS ET AL.	
	Examiner	Art Unit	
	Leah Schlientz	1618	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *23 July 2009*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-4,6-8 and 11* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-4,6-8 and 11* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *3/5/2009*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Acknowledgement of Receipt

Applicant's Response, filed 7/23/2009, in reply to the Office Action mailed 6/24/2009, is acknowledged and has been entered. Claims 1-4, 6-8 and 11 have been amended. Claims 5, 9, 10, 12 and 13 have been cancelled. Claims 1-4, 6-8 and 11 are pending and are examined herein on the merits for patentability.

Information Disclosure Statement

The information disclosure statement (IDS) submitted on 3/5/2009 was filed after the mailing date of the Office Action on 12/9/2008. The submission is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statement is being considered by the examiner.

Response to Arguments

Any rejection or objection not reiterated herein has been withdrawn as being overcome by amendment.

Applicant's arguments have been fully considered but are moot in view of new grounds for rejection set forth hereinbelow, necessitated by amendment.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-4, 6-8 and 11 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The claims are drawn to a method of optical imaging of oesophageal cancer or Barrett's oesophagus involving administering a contrast agent with an affinity for an abnormally expressed biological target associated with oesophageal cancer or Barrett's oesophagus, wherein the biological target is selected from E-cadherin, CD44, P62/c-myc (HGF receptor), p53 and EGFR/erB-2. Dependent claims recite a contrast agent of formula I, V-L-R, wherein V is one or more vector moieties having affinity for an abnormally expressed target in oesophageal cancer or Barrett's oesophagus, L is a linker moiety or a bond and R is one or more reporter moieties detectable in in vivo optical imaging. In dependent claims the contrast agent has a molecular weight below 14000 Daltons. However, the claims are devoid of any structural elements that correlate to the function which is to be achieved with the claimed composition. For example, a vast number of potential "vector moieties having an affinity for an abnormally expressed target in oesophagael cancer or Barrett's oesophagus" may be found in the art to be

capable of having the claimed function. Applicant has identified in the instant specification a diverse variety of targets for which the vector may have affinity including E-cadherin, CD44, P62/c-myc (HGF receptor), p53 or EGFR/erB-2, etc. among others (see paragraphs 0026-0048 of the instant specification). Such targets are widely varying in structure and would have an almost unlimited number of potential vectors which may have affinity thereto. The vectors themselves may be almost unlimited including various peptide sequences, small molecules, antibodies, nucleic acid sequences, etc. It is clear that Applicant had possession of such a few specific formulations at the time of filing using specific and defined vectors as identified in paragraphs 0061-0066 and the Examples, but the specification as originally filed does not provide support that Applicant had possession of the invention as generically claimed by function alone in the instant claims. For example, to arrive at the claimed contrast agent, one would have to determine the type of vector having affinity to which out of an extremely large number of targets to conjugate to which out of an almost unlimited number of potential optical imaging moieties to be combined into a single agent, and further which out of an almost unlimited number of potential functional groups or chemical reactions would be necessary to derivatize and conjugate the moieties into a single agent having the claimed functional properties. One would have to select which portions of which molecules would be suitable to be conjugated to the others and on what positions of the molecules with various substituents. Applicant's limited disclosure of a particular compound which has the claimed functional properties does not provide support that Applicant envisaged the

invention as a whole which is broadly claimed solely by function. In the instant case, a definition by function alone does not appear to sufficiently describe the claimed invention because it is only an indication of what the agent does, rather than what it is. See MPEP 2163 and *Eli Lilly,* 119 F.3 at 1568, 43 USPQ2d at 1406.

Applicant argues on page 5 of the Response that claim 1 has been amended to include the elements of previous claims 10 and 5, and is no longer to optical imaging contrast agents *per se.* Hence, it can no longer be argued that the claim pertains to compounds defined only by their function. In addition, the claim scope has been limited to the five biological targets of previous claim 5. Applicants contend that the specification provides sufficient information for the person skilled in the art to reproduce the method of amended claim, and that the person skilled in the art can either use the contrast agents described in the specification, or generate new ones. Applicants suggest that the claim scope for such an optical imaging method claim should not be limited by the possible future advent of new targeting molecules. If a person skilled in the art has available a compound with affinity for E-cadherin, CD44, P62/c-myc (HGF receptor), p53 or EGFR/erB-2, then labelling such a compound with an optical reporter is taught by the present specification.

This is not found to be persuasive. In order to practice the claimed method, one would necessarily be in possession of the contrast agent, thus a reasonable description of the contrast agent which are used to practice the method is necessary. While Applicant has provided a description of a few

specific vectors (i.e. a single peptide sequence which binds p53 (paragraph 0061), a 4-anilinquinazoline compound which targets EGFR/erB-2 (paragraph 0062). Such a limited disclosure of a single vector for each of the claimed receptors which are associated with esophageal cancer or Barrett's esophagus does not provide sufficient description to show that Applicant was in possession of the full scope of a contrast agent comprising an optical imaging moiety and any vectors (e.g. any small molecule, any peptide, any oligonucleotide, any antibody etc) which may target the claimed receptors. With regard to Applicant's argument that the claim scope should not be limited by the possible future advent of new targeting molecules, and that if a person skilled in the art has available a compound with affinity for one of the targets described, then labeling such a compound with an optical reporter is taught by the specification, this is not found to be persuasive because the specification has not provided a clear description of the full scope of targeting vectors which were envisaged at the time the specification was filed. Future-developed targeting moieties would not be encompassed by vectors that Applicant was in possession of at the time the application was filed, especially since Applicant has only described a single vector for each target/receptor. In the instant case, a definition by function alone does not appear to sufficiently describe the claimed invention because it is only an indication of what the agent does, rather than what it is. See MPEP 2163 and *Eli Lilly,* 119 F.3 at 1568, 43 USPQ2d at 1406. The claims are more broad than the enabling disclosure. For example, with regard to the vector moiety the

claims identify the moiety by what it does (i.e. targets p53, for example), rather than what it is (i.e. a structurally defined peptide sequence such as Cys-Gly-Pro-Leu-Gly-Leu-Leu-Ala-Arg-OH).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

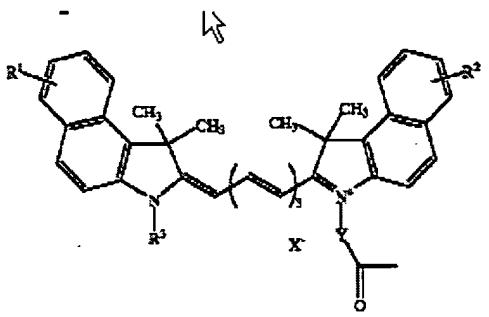
> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

Claims 1, 3, 7, 8 and 11 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ito et al. (US 5,968,479) in view of Iversen (US 6,365,577).

Ito discloses a diagnostic marker containing (a) a detection system such as an antibody and (b) a fluorescent functional group that is bound to the detection system and represented by the formula:



The diagnostic marker emits fluorescence having a wavelength of 780 nm or more when irradiated with near or far infrared rays, and thus useful for infrared endoscopic diagnosis or identification of a focus in surgical operation (abstract). As pharmacologically and pharmaceutically acceptable additives for the manufacture of the diagnostic agent of the present invention, for example, excipients, disintegrators, etc. (column 11, lines 23+). Targeting to p53 protein for *in vitro* and *in vivo* targeting of cancer of the stomach is disclosed (column 9, lines 10-30).

Ito does not specifically recite imaging esophageal cancer via p53 targeting.

Iversen discloses that preferred target cells are cancer cells characterized by p53 expression. Such cancers include those of bladder, brain, breast, cervix, colon, esophagus, larynx, liver, lung, ovary, pancrease, prostate, skin, stomach and thyroid.

It would have been obvious to one of ordinary skill in the art at the time of the invention to perform optical imaging of esophageal cancer upon administration of the p53 antibody-fluorescent compound conjugates disclosed by Ito. While Ito does not specifically recite that esophageal cancer is imaged

with his compositions, i is known in the art that p53 is overexpressed in both esophageal cancer, as well as stomach cancer, as shown by Iversen. It would have been further obvious to image/diagnose esophageal cancer in order to expand upon the cancer types for which the conjugates of Ito are useful. One would have had a reasonable expectation of success in doing so because Iversen teaches the correlation between erbB-2 and esophageal cancer.

Claims 1-4, 6-8 and 11 are rejected under 35 U.S.C. 103(a) as being unpatentable over Quinn *et al.* (US 7,585,509) in view of Chiang *et al.* (*Clin. Cancer Res.*, 1999, 5, p. 1381-1386).

Quinn discloses a method for targeting an agent to a **cancer cell expressing ErbB-2** comprising bringing said cancer cell into contact with a peptide-agent complex, wherein said peptide comprises the sequence KCCYSL (SEQ ID NO:1) and said peptide binds to the extracellular domain of ErbB-2, wherein said agent is a **diagnostic agent**, a chemotherapeutic, a radiotherapeutic, a toxin or a cytokine (claim 1). The diagnostic agent may be a **fluorescent label** (claim 3). The complex further comprises a linking moiety that connects said agent and said peptide (claim 13). There are provided methods for diagnosing ErbB-2-positive cancer in a subject comprising (a) administering to the subject a peptide-diagnostic agent complex, wherein the peptide comprises the sequence KCCYSL; and (b) assessing the amount and/or localization in the subject, of the diagnostic agent. The diagnostic agent may be a radiolabel, a chemilluminescent label, a fluorescent label, a magnetic spin

resonance label, or a dye. The patient may or may not have been previously diagnosed with cancer. The patient may have previously received a cancer therapy, or may be concurrently receiving a cancer therapy. The may be patient at an elevated risk for cancer. The assessing may comprise organ or whole body imaging, and may further comprise excising a tumor localized by the diagnostic agent (column 2, lines 38+). **Fluorescent labels contemplated for use as conjugates** include Alexa 350, Alexa 430, AMCA, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-TRX, Cascade Blue, Cy3, Cy5,6-FAM, Fluorescein, Isothiocyanate, HEX, 6-JOE, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, REG, Rhodamine Green, Rhodamine Red, Renographin, ROX, TAMRA, TET, Tetramethylrhodamine, and/or Texas Red (column 10, lines 42-50).

Quinn does not specifically teach that that esophageal cancer is imaged.

Chiang discloses that about 20% of esophageal adenocarcinomas show amplification of the *erbB*-2 oncogene, and that the capability to detect abnormalities in serum of esophageal cancer patients creates an opportunity to diagnose esophageal cancer and to monitor the outcome of treatment (page 1381).

It would have been obvious to one of ordinary skill in the art at the time of the invention to perform optical imaging of esophageal cancer upon administration of the erbB-2 receptor targeting peptide conjugates disclosed by Quinn. While Quinn does not specifically recite that esophageal cancer is imaged with his compositions, it is taught that erbB-2 is overexpressed in a

number of cancer types including those involving the female genital tract (e.g., endometrial cancer), gastric cancer and prostate cancer, and that a primary target for ErbB-2 targeted therapies is breast cancer, of which 20-30% show overexpression of this marker (column 30, lines 35+). It is known in the art that erbB-2 is also overexpressed in esophageal cancer, as shown by Chiang. Since Quinn generally teaches that his compositions are useful for diagnosing erbB-2-positive cancer in a subject (column 2, claim 1), and that erbB-2 is overexpressed in a number of cancer types, it would have been further obvious to image/diagnose esophageal cancer in order to expand upon the cancer types for which the conjugates of Quinn are useful. One would have had a reasonable expectation of success in doing so because Chiang teaches the correlation between erbB-2 and esophageal cancer.

Conclusion

No claims are allowed at this time.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, THIS ACTION IS MADE FINAL. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory

period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Leah Schlientz whose telephone number is 571-272-9928. The examiner can normally be reached on Monday - Friday 8 AM - 5 PM. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Michael Hartley can be reached on 571-272-0616. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Michael G. Hartley/
Supervisory Patent Examiner, Art Unit 1618

LHS

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (11-08)
Approved for use through 12/31/2008. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99**)		
	Application Number	10582679
	Filing Date	2007-05-17
	First Named Inventor	Jo Klaveness
	Art Unit	1618
	Examiner Name	Leah H. Schlientz
	Attorney Docket Number	PN0397

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	6630570		2003-10-07	LICHA ET AL.	

If you wish to add additional U.S. Patent citation information please click the Add button.

U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	WO 1998/047541	WO		1998-10-29	NYCOMED IMAGING AS		☐
	2	WO 2000/071162	WO		2000-11-30	MALLINCKRODT INC.		☐
	3	WO 2001/089584	WO		2001-11-29	NYCOMED IMAGING AS		☐

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /LS/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	10582679
	Filing Date	2007-05-17
	First Named Inventor	Jo Klaveness
	Art Unit	1618
	Examiner Name	Leah H. Schlientz
	Attorney Docket Number	PN0397

	4	WO 2001/091805	WO		2001-12-06	BRACCO RESEARCH USA		☐
	5	WO 2005/002293	WO		2005-01-06	VANDERBILT UNIVERSITY		☐
	6	WO 2005/030266	WO		2005-04-07	AMERSHAM HEALTH AS		☐
	7	EP 1 170 021	EP		2002-01-09	SHERING AKTIENGESELLSCHAFT		☐
	8	EP 0 800 831	EP		2004-04-21	DAIICHI PURE CHEMICALS CO. LTD.		☐

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NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1		☐

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EXAMINER SIGNATURE

Examiner Signature	/Leah Schlientz/	Date Considered	06/08/2009

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /LS/

Notice of References Cited	Application/Control No. 10/582,679	Applicant(s)/Patent Under Reexamination KLAVENESS ET AL.	
	Examiner Leah Schlientz	Art Unit 1618	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-7,585,509	09-2009	Quinn et al.	424/195.11
*	B	US-6,365,577	04-2002	Iversen, Patrick L.	514/44A
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Chiang et al., Clin. Cancer Res., 1999, 5, p. 1381-1386.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Detection of *erbB*-2 Amplifications in Tumors and Sera from Esophageal Carcinoma Patients[1]

Pei-Wen Chiang,[2] David G. Beer, Wan-Li Wei, Mark B. Orringer, and David M. Kurnit

Departments of Pediatrics [P-W. C., W-L. W., D. M. K.], Surgery [D. G. B., M. B. O.], and Human Genetics [D. M. K.], University of Michigan Medical Center, Ann Arbor, Michigan 48109-0650

ABSTRACT

We used TaqMan PCR to detect quantitative anomalies of tumor markers in both tumor and serum DNA from esophageal cancer patients. We demonstrated the potential of this methodology by detecting *erbB*-2 amplifications in a plurality of esophageal tumor samples. These amplifications were corroborated by Southern blots. We then showed the potential of this methodology to detect quantitative anomalies of *erbB*-2 in serum DNA from individuals with a corresponding amplification in the tumor. The capability of TaqMan PCR to detect abnormalities in serum of esophageal cancer patients creates an opportunity to diagnose esophageal cancer and to monitor the outcome of treatment with a blood test.

INTRODUCTION

Recent studies indicate that soluble tumor DNA is found in the serum and plasma of cancer patients; alterations of both microsatellite and specific sites corresponding to the lesions in tumors have been identified in the serum or plasma of patients with several cancers (1–5). Microsatellite analysis of serum represents a noninvasive method for the detection of circulating tumor cell DNA. This encouraged us to determine whether such gene copy alterations could be detected in an analogous manner by another noninvasive molecular methodology, TaqMan PCR. As a model system, we followed the amplification status of *erbB*-2 in esophageal tumors.

The incidence of esophageal adenocarcinoma has increased markedly in the past two decades (6). The mortality associated with esophageal adenocarcinoma is high because many tumors are not detected until the disease has progressed to an advanced stage. Even when the primary tumor is resectable, the overall 5-year survival rate is still low (7). New approaches to accomplishing early detection and to monitoring the course of therapy would benefit the clinical management of this disease.

Received 11/23/98; revised 2/22/99; accepted 2/24/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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About 20% of esophageal adenocarcinomas show amplification of the *erbB*-2 oncogene (8).[3] Amplification of oncogenes in cancer can be detected by either quantitative Southern blotting (9), which requires large amounts of DNA, or by FISH[4] (10, 11), which requires intact cells. However, neither of these techniques can be used to monitor changes in the small amounts of acellular DNA present in serum. Deletion of tumor suppressor genes in cancer can be detected by LOH analysis (12), but it requires polymorphic markers. Recently, we developed a fluorescent QPCR assay that can monitor gene copy number variation in cancer genomes (13, 14). Fluorescent QPCR has the following advantages over Southern blotting, FISH, and LOH studies: (*a*) both gene amplifications and deletions can be detected by fluorescent QPCR; (*b*) only a small amount of DNA is needed for this assay, so the small amounts of DNA that can be extracted from sources like serum and urine sediment are suitable for this assay; (*c*) only DNA (and not intact cells) is required for analysis, permitting analysis of acellular fluids, such as serum, that can still contain DNA from other sources; (*d*) no polymorphic markers are required for the assay, so all markers are informative in all individuals; and (*e*) pairing of an oncogene marker that is often amplified (*e.g.*, *erbB*-2) and a tumor suppressor marker that is often deleted (*e.g.*, *p16* or *p53*) maximizes the ability of the QPCR technique to detect quantitative alterations seen in cancer but not in normal cells. Here, we show that the sensitive and facile TaqMan PCR technique can detect *erbB*-2 amplifications in both tumors and sera of individuals with adenocarcinoma of the esophagus. Although such quantitative alterations occur only in a plurality of tumors, the study indicates a proof of principle that this technique is a valuable addition to the way cancer is monitored.

MATERIALS AND METHODS

Principle of TaqMan Assay. The TaqMan assay has been described previously (15–17). Briefly, the assay is based on the principle that successful PCR yields a fluorescent signal due to Taq-mediated exonuclease digestion of a fluorescence-labeled oligonucleotide homologous to a sequence between the two primers. The extent of digestion, which depends directly on the amount of PCR that occurs, can be quantified directly and accurately by measuring the increment in fluorescence that results from decreased energy transfer. This sensitive measurement allows detection in the exponential phase of the PCR, which is required for determination of initial genomic sequence copy number.

Mechanically, this was accomplished by performing the assay on an aliquot of the esophageal tumor or serum DNA. The

[3] D. G. Beer, unpublished data.

[4] The abbreviations used are: FISH, fluorescent *in situ* hybridization; LOH, loss of heterozygosity; QPCR, quantitative PCR; nt, nucleotide(s).

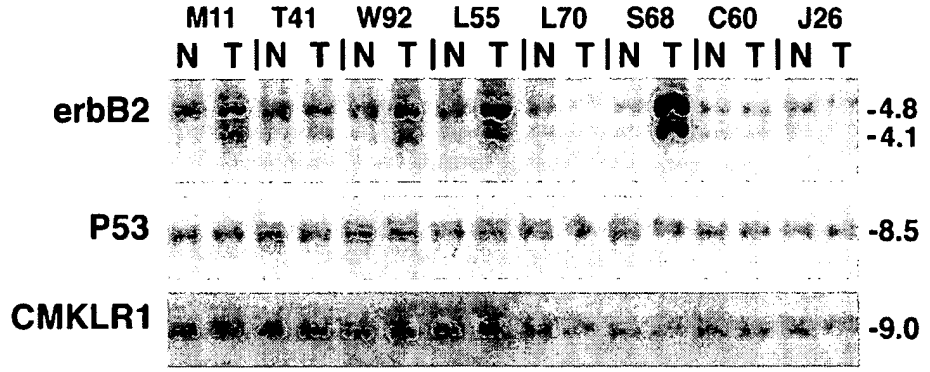

Fig. 1 Southern blot determination of *erbB*-2 and *p53* in esophageal cancer. Ten μg of DNA were obtained from esophageal tumor or normal control tissue (normal esophagus or gastric tissue). The resultant DNA was digested with *Eco*RI and separated on 0.9% agarose gels. The blots were probed, stripped, and rehybridized with probes for *erbB*-2, *p53*, and the control gene, *CMKLR1*.

relative gene copy number of two sequences was derived from the ratio of TaqMan PCR for each of these sequences. The oligonucleotides and primer for a given sequence were added to the reaction mixture containing DNA from the sample of interest in the presence of uracil DNA glycosylase (18) and AmpliTaq Gold (Perkin-Elmer Corp., Norwalk, CT), per the manufacturer's suggestion. Any carryover DNA was eliminated by uracil DNA glycosylase in a 2-min treatment at 50°C. Following a subsequent 10-min incubation at 95°C to denature the input DNA, the DNA was PCR-amplified using AmpliTaq Gold in a two-step amplification (15 s at 95°C to denature the DNA and 1 min at 60°C to permit annealing and elongation). To assay the initial concentration of the reactants (the gene copy number), we measured the number of cycles at which the reaction crosses a threshold value. This number, C_T, varied directly with the initial gene copy number. To measure the relative genomic copy numbers of two sequences in a given DNA sample, we divided the C_T derived using the first sequence with the given DNA sample by the C_T value using the second sequence. Derivation of this fraction was independent of DNA sample concentration, eliminating the requirement to measure DNA concentration accurately. As a result, an abnormal value of ≥ 2 could result from amplification of the first sequence and/or deletion of the second sequence. The contribution of each probe to the value could also be determined by evaluating additional probes, if desired, but this was not required for the TaqMan PCR assay.

DNA Preparation. DNA from normal leukocytes was purchased from Boehringer Mannheim. DNA from normal gastric tissue, normal esophagus, Barrett's mucosa, and esophageal adenocarcinoma (either Barrett's adenocarcinoma or adenocarcinoma arising from the cardia) were prepared as described (8). The normal and tumor tissues were removed from patients undergoing esophagectomy and designated by histological criteria as tumorous tissue, Barrett's metaplasia, normal-appearing gastric mucosa, and normal-appearing esophageal mucosa. Preparation of serum DNA was accomplished by a kit from Biotronics (Lowell, MA). This DNA was collected from patient samples obtained at the time of surgery for esophageal cancer, Barrett's mucosa with high-grade dysplasia, achalasia, or stricture.

PCR Primers for Measurement of Genomic Sequence Copy Number. The sequences of primers for *erbB*-2, *p53*, and *p16* were: (*a*) *erbB-2*: TaqMan probe, 6-FAM-5′-AGAGGGCCCTCTGCCTGCTGC-3′-TAMRA; erb2U18 (nt 252), 5′-AGGGAGTGGCAGAGACAC-3′; and erb2L17 (nt 528), 5′-GGCTGAAGGCAGGAGGA-3′; (*b*) *p53*: TaqMan probe, 6-FAM-5′-AGCAGCTCCTACACCGGCGGC-3′-TAMRA; p53U19 (nt 11,985), 5′-CCTGGTCCTCTGACTGCTC-3′; and p53L19 (nt 12,227), 5′-GTAGCTGCCCTGGTAGGTT-3′; and (*c*) *p16*: TaqMan probe, 6-FAM-5′-CTGCTGCTGCTCCACGGCGC-3′-TAMRA; p16U20 (nt 15), 5′-GGAAGCAAATGTAGGGGTAA-3′; and p16L17 (nt 267), 5′-CCAGCGTGTCCAGGAAG-3′.

Quantitative Southern Blotting. Quantitative Southern blot analysis of the *erbB*-2 and *p53* genes was performed as described previously (8). Southern blots were stripped after hybridization to erbB-2 or p53 and rehybridized with the single copy control gene, *CMKLR1* (19). The blots were quantified by scanning laser densitometry using an Image Quant Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). The ratio of *erbB*-2/CMKLR1 or p53/CMKLR1 was used to quantitate *erbB*-2 or *p53* in normal, Barrett's metaplasia, and esophageal cancer samples.

RESULTS

Evaluation of TaqMan QPCR Systems for *erbB*-2, *p53*, and *p16* Markers. The resolution, sensitivity and reproducibility of TaqMan PCR was determined by performing TaqMan PCR on 2-fold dilution series of duplicated normal human DNA in the range from 20 ng to 24 pg. Three primer sets derived from the *erbB*-2, *p53*, and *p16* genes were tested in this analysis. The result confirmed that the TaqMan PCR values were halved as expected for each 2-fold dilution of the DNA. This was true down to 24 pg for the erbB-2 and p16 probes but only down to 1 ng for the p53 probe. This documents that the assay is sensitive enough to detect 2-fold concentration differences of DNA. The values duplicated for replicate samples performed for a given probe each time we ran the assay. With a maximum variation of 50% between duplicate points, the TaqMan PCR values yielded satisfactory results for each of the three probes with normal human DNA at every dilution.

We also checked the accuracy of TaqMan PCR by comparing TaqMan PCR results with previous Southern blotting. Using Southern blotting, we quantitated copy number of the oncogene, *erbB*-2, and the tumor suppressor gene, *p53*, in "nor-

Table 1 Copy number ratios in 39 normal samples and 39 tumor samples

Tissue source (no. of cases)	Taq Man PCR (*erbB-2/p53*)	Southern (*erbB-2*)
Barrett's metaplasia (2)		
1	0.8	
2	1.3	
Mean ± SD	1.1 ± 0.4	
Range	0.8–1.3	
Gastric, normal (9)		
1	0.9	
2	1.0	
3	1.0	
4	1.1	
5	1.1	
6	1.1	
7	1.1	
8	1.1	
9	1.2	
Mean ± SD	1.1 ± 0.1	
Range	0.9–1.2	
Esophagus, normal (28)		
1	0.7	
2	0.8	
3	0.8	
4	0.8	
5	0.8	
6	0.8	
7	0.8	
8	0.9	
9	0.9	
10	0.9	
11	0.9	
12	0.9	
13	0.9	
14	1.0	
15	1.0	
16	1.0	
17	1.0	
18	1.0	
19	1.1	
20	1.2	
21	1.3	
22	1.3	
23	1.4	
24	1.5	
25	1.7	
26	1.8	
27	1.8	
28	2.0	
Mean ± SD	1.1 ± 0.4	
Range	0.7–2.0	
Tumors (39)		
1	0.8	1
2	0.9	1
3	0.9	1
4	1.0	1
5	1.0	1
6	1.0	1
7	1.0	1
8	1.1	1
9	1.1	1
10	1.1	1
11	1.1	1
12	1.2	1
13	1.2	1
14	1.3	1
15	1.3	1
Tumor		
16	1.4	1
17	1.5	1
18	1.6	1
19	1.6	1
20	1.6	1
21	1.7	1
22	1.7	1
23	1.8	1
24	1.8	1
25	2.9 ± 0.3[a]	1.8
26	3.0 ± 0.4[a]	2.6
27	3.7 ± 0.2[a]	1
28	4.2 ± 0.2[a]	2.5
29	4.5 ± 0.6[a]	1
30	6.7 ± 0.2[a]	1
31	7.0 ± 2.7[a]	1
32	13.4 ± 1.8[a]	5.6
33	13.6 ± 1.6[a]	5.6
34	16.8	18.4
35	17.1	7.7
36	26.2	5.8
37	51.5	15.6
38	94.6 ± 9.2[b]	50.6
39	106.7 ± 17.6[b]	20

[a] Mean ± SD, two assays.
[b] Mean ± SD, four assays with dilution series.

mal" and "esophageal tumor" tissues (Fig. 1). The amount of *erbB-2* or *p53* on the blots was normalized against the single copy gene, *CMKLR1*. Using TaqMan PCR, we then quantitated the ratio of the copy numbers of *erbB-2/p53* in these 39 normal samples and 39 tumor samples (Table 1).

The *erbB-2/CMKLR1*, *p53/CMKLR1* (by Southern blotting), and *erbB-2/p53* (by TaqMan PCR) ratios were ~1 (between 0.6 and 1.9) for controls. However, in tumors, amplifications of the oncogene *erbB-2* copy number relative to the tumor suppressor *p53* copy number were observed by both Southern blotting and TaqMan PCR (Table 1). Fifteen of the 39 tumors were abnormal, with a TaqMan PCR value of *erbB-2/p53* ≥2 (Table 1). For the lowest nine values among the TaqMan PCR values of ≥2 in Table 1, we repeated the TaqMan PCR analysis and confirmed that an amplification of *erbB-2/p53* of ≥2 was observed again. The means and SDs for these analyses are given (Table 1). In 11 of these 15 cases, the Southern blots also showed a copy number amplification of *erbB-2*. In four of the cases with smaller amplifications upon repeated TaqMan PCR, the Southern blots with *erbB-2* did not detect an anomaly, consistent with the known difficulty using a single Southern blot to detect smaller quantitative alterations (10). The duplication of TaqMan PCR for these four samples with low degrees of *erbB-2* amplifications verifies the increased sensitivity of TaqMan PCR to detect lower level amplifications. The ability of the TaqMan PCR technique to assay smaller amplifications that are ≥2-fold variations from the norm is substantiated by our extensive control data on both normal DNA and on dilutions of tumor DNA (Table 1; see above). These data

Table 2 Derivation by TaqMan PCR *erbB-2/p16* from serum and from tumors in individuals with esophageal cancer and abnormal serum values

Sample no.	Diagnosis[a]	No. of cases	TaqMan PCR	
			Serum	Tumor
Nonmalignant esophageal lesion		5	0.8–1.5 (1.1)[b]	
77	Achalasia, megaesophagus		0.8	
52	Stricture		1.0	
41	Staple exclusion		1.1	
58	Hiatal hernia		1.3	
73	Epiphrenic diverticulum		1.5	
Barrett's metaplasia of esophagus		5	0.8 –1.7 (1.2)[b]	
45	Barrett's, severe esophagitis		0.8	
64	Barrett's		0.9	
50	Barrett's with dysplasia		1.3	
67	Barrett's with high-grade dysplasia		1.6	
74	Barrett's with high-grade dysplasia		1.7	
Malignancy of esophagus (normal serum), pretreated with anticancer therapy		11	0.9–1.7[c]	0.4–1.8
55	III/adeno/cardia		0.9	0.4
53	III/squamous cell/lower		1.0	
70	III/adeno/cardia		1.1	0.6
66	IIB/adeno/middle		1.1	1.4
71	IIA/adeno/lower		1.1	1.4
44	IIA/adeno/lower		1.3	1.8
48	I/adeno/lower		1.3	0.9
54	I/adeno/lower		1.4	1.5
59	III/adeno/cardia		1.4	1.1
78	IIB/adeno/lower		1.7	
79	III/adeno/lower		1.7	0.4
Malignancy of esophagus (normal serum); not pretreated		14	0.7–1.6[c]	0.4–34.3[c]
80	IIB/adenol/cardia		0.7	34.3
47	IIA/adeno/cardia		0.9	1.8
46	I/adeno/lower		1.0	
69	I/adeno/cardia		1.0	1.0
42	III/adeno/lower		1.1	0.7
49	IIA/squamous cel/lower		1.1	1.4
56	IVA/adeno/cardia		1.1	
61	IIA/adeno/cardia		1.2	1.0
63	I/adeno/lower		1.2	
51	III/adeno/lower		1.4	1.6
65	IIB/adeno/cardia		1.4	0.5
43	IVA/adeno/cardia		1.5	0.8
75	III/adeno/lower		1.6	0.4
68	I/adeno/lower		1.6	
Malignancy of esophagus (abnormal serum), not pretreated		5	2.0–3.8[c]	2.5–8.9[c]
57	III/squamous cell/middle		2.0	2.5
60	I/adeno/cardia		2.2	7.9
72	III/adeno/lower		2.5	8.9
62	IIA/adeno/lower		3.7	3.1
76	IVA/adeno/lower		3.8	NA[d]

[a] Slashes represent stage/type/anatomic location. adeno, adenocarcinoma.
[b] Values represent ranges (means).
[c] Not including the tumor with a TaqMan PCR value of 34.3.
[d] NA, not available.

document the ability of the TaqMan PCR assay to distinguish 2-fold differences.

Detection of Abnormality in the Serum DNA from Esophageal Adenocarcinoma Patients. Given the small amount of DNA required for TaqMan PCR, we applied this technique to the analysis of DNA in serum. There was significantly less DNA in serum than available from tumor samples, averaging ~100 copies per reaction. At these low concentrations of DNA, our *erbB*-2 probe was appropriately sensitive for TaqMan PCR, but the *p53* probe was not. The probe for p16, however, was sufficiently sensitive to work at these low DNA concentrations. As noted above, this probe satisfied all of the requirements met by the *erbB*-2 probe, showing a good 2-fold discrimination between DNA samples at low concentrations, down to 24 pg.

Therefore, we used the probe pairing of *erbB*-2 and *p16* to screen 39 sera blindly (Table 2; these sera were from different patients than the tumor DNAs that were analyzed in Table 1 for

erbB-2/p53). We also did the corresponding tumor DNA with *erbB-2* and *p16* when the tumor was available. In five cases, the esophageal lesion was nonmalignant, and in another five cases, the sera was from patients with premalignant Barrett's metaplasia or Barrett's metaplasia with dysplastic changes. In each of these 10 cases, the serum TaqMan PCR was normal (<2). We detected amplifications ≥2 of the *erbB-2*/p16 ratio in five serum samples from 19 subjects with untreated esophageal tumors. Presumably, all five abnormal serum samples corresponded to an untreated tumor with *erbB-2* amplification (in four, the tumor showed an amplification of *erbB-2*; and in one, the tumor was unavailable but presumably had such an amplification). There were two tumors with normal serum *erbB-2/p16* ratios that showed a deletion equivalent to one copy of *erbB-2*, and one tumor that showed a large amplification of *erbB-2*. In neither case did we see a corresponding quantitative anomaly of serum *erbB-2/p16*. In addition, we did not observe anomalies of the serum in the remaining 11 samples from subjects who had been treated by radiotherapy and/or chemotherapy prior to obtaining the sample (two of whom showed a deletion of *erbB-2/p16* in the tumor).

DISCUSSION

Accuracy of the TaqMan Methodology. Although competitive PCR technologies have been advanced to perform quantitation (20, 21), they are difficult to perform with great facility and accuracy. In contrast, the facile TaqMan PCR technology (15–17) affords a real-time assay. Our extensive control data permit the demarcation of abnormal TaqMan PCR values to be ≥2. When the TaqMan PCR technique was applied to normal leukocyte DNA, the variation we observed was routinely <50% between replicate samples. In no case did we observe a TaqMan PCR value for this normal DNA of <0.7 or >1.3 times the expected value. Furthermore, only 5 of 39 esophageal samples that were classified as normal by histological criteria had TaqMan PCR values between 1.4 and 1.9 (*i.e.*, showed a deviation of >50% from a normal value of 1; the single normal sample with a value of 2.0 came from an esophagus in which the tumor had a very high value of 16.8). Indeed, we saw a lower range of high TaqMan PCR values with known normal standard DNA than DNA from these normal esophageal samples taken from subjects with known esophageal cancer. These high TaqMan PCR values could reflect contamination of normal cells with malignant cells, small increases of gene copy number in premalignant cells. or unsuspected difficulties arising during DNA purification.

In all 11 cases in which Southern blotting detected an amplification of *erbB-2*, the TaqMan PCR value was abnormally elevated. However, the amplifications seen on the Southern blot for *erbB-2* were frequently less than the changes seen in TaqMan PCR (Table 1). Given the reproducibility of TaqMan PCR, the most likely explanation for this discrepancy is that Southern blotting is less sensitive and underestimates the true value of *erbB-2* amplification. In particular, there were four cases in which the TaqMan PCR values were abnormally elevated but the Southern blots did not show quantitative changes. The effect of p53 deletion could not fully explain the difference in these four cases. This apparent insensitivity of the Southern blotting method reflects the difficulty of this method to detect small variations without multiple repetitions (10). A similar determination that TaqMan PCR represents a suitable methodology to ascertain amplifications comes from two recent analyses of amplification events in breast cancer (22, 23). In summary, compared with Southern blotting, TaqMan PCR has the dual advantages of increased sensitivity and the ability to use both deletions and amplifications because our usage depends on the ratio between a tumor suppressor gene and an oncogene (Table 1).

Analysis of Serum for Monitoring the Patient with Cancer. A major contribution of this analysis is the potential to detect amplifications in serum that result from cancer. Stroun *et al.* (24) showed that there was a small amount of free DNA in serum due to death of normal nucleated blood cells and/or vessel wall endothelial cells. The lysed genome of tumor cells that outstrip their blood supply and/or represent the products of metastatic cells are added to this serum DNA content from normal cells. On the basis of this, a variety of tumors can yield LOH or novel restriction enzymes digestion sites detectable in serum DNA (1–5). The finding that tumor DNA was sometimes present in the serum led us to detect serum DNA quantitative alterations by TaqMan PCR. The sensitivity of TaqMan PCR coupled with the lack of requirement for intact cells (as in FISH) could make this a worthwhile approach to monitoring the serum for both detection and following therapy of cancer.

Analysis of serum with the probes *erbB-2* and *p16* is detailed in Table 2. For this task, our p53 probe was not sufficiently sensitive, so we had to use our p16 probe instead to monitor the small amount of DNA in serum along with the *erbB-2* probe. All 10 sera from subjects who did not have esophageal cancer had normal serum QPCR values of <2, as expected. All 11 sera from patients who had preoperative chemotherapy and/or radiotherapy, in whom tumor size would be expected to be minimal, did not show abnormal serum values. Five sera of 19 from individuals with esophageal tumors that were not pretreated, including 5 of 6 sera with corresponding elevated tumor values of the *erbB-2/p16* ratio, were abnormally elevated; *i.e.*, as they showed TaqMan PCR values of ≥2. Thus, five of six untreated tumors could be detected by serum analysis, reflecting bulky tumors outstripping their blood supply and/or hematogenous dissemination (1–5). Presumably, the one tumor with an elevated *erbB-2/p16* ratio that did not show an elevated serum *erbB-2/p16* ratio had not seeded the serum. Our results indicated that TaqMan PCR could be used to monitor cancer therapy by serum analyses. Although this series is too small to draw definite conclusions, it is of interest to note that the serum was positive with tumors of different stages. Although the ultimate clinical significance will require larger studies, this suggests the feasibility of monitoring serum by the facile QPCR technology.

The inability to detect a loss of erbB-2/p16 and the finding of cases in which the serum QPCR values are less than the tumor QPCR values both indicate that normal cellular DNA in the serum (24) can dilute esophageal tumor DNA in the serum. In this light, screening for QPCR anomalies with greatest quantitative differences will maximize the sensitivity of this test. The failure to find LOH anomalies in serum of colon cancer subjects

(5) underscores that the phenomenon of serum abnormalities in cancers can be tumor specific.

In the tumor, normal DNA is contributed by normal cells in the biopsy specimen; in the serum, normal DNA is contributed by lysis of normal blood and endothelial cells. This explains why there is discordance between tumor and serum QPCR values even when hematogenous seeding by tumor cells has occurred. Thus, although this study underscores the potential usefulness of using the simple, rapid, and inexpensive technique of TaqMan PCR to monitor serum, questions regarding the sensitivity of the technique in this and other malignancies remain to be addressed. Although this represents only a small series of relevant patients who were not pretreated and who had significant amplification of *erbB*-2, it indicates the potential usefulness of monitoring QPCR in serum of patients with amplifications of QPCR in the tumor.

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